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Bacterial Endotoxic Lipopolysaccharides

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Chapter 18

LIPID A AND LIPOSOMES CONTAINING LIPID A AS ADJUVANTS FOR VACCINES

Carl R. Alving

TABLE OF CONTENTS

I. ADJUVANT ACTIVITIES OF LIPOPOLYSACCHARIDE AND LIPID A

The ability of Gram-negative bacterial lipopolysaccharide (LPS) to serve as an adjuvant to enhance immune responses to antigens has been recognized for more than 30 years.[1-3] LPS itself is also highly immunogenic. As evidence of this it was shown that LPS could sensitize mice to a secondary immune response with a few tens of molecules (picogram amounts)[4] and could induce primary immune responses in rabbits after injection of only a few thousand molecules.[5] Because of its potent immunogenicity LPS has been referred to as a "super antigen."[4] Adjuvant activity of LPS was subsequently shown to reside in the lipid A moiety, and it was hypothesized that "LPS is a powerful immunogen because it is an antigen [polysaccharide] that carries its own adjuvant [lipid A]."[6]

The adjuvant activity of lipid A has been confirmed in many laboratories,[7-11] and activity has also been demonstrated with synthetic analogs of lipid A.[12-14] Experiments with chemically modified LPS have led to the conclusion that only the lipid A portion is required for adjuvanticity, and adequate amounts of esterified and amidated lipid A fatty acids are also required.[8]

II. MECHANISMS OF ADJUVANT ACTIVITY

A. INSIGHTS FROM SYNTHETIC LIPID A ANALOGS

The availability of synthetic lipid A analogs has permitted analysis of minimal structural requirements for adjuvant activity.[15,16] Synthetic analogs have also provided insights into the relatively small importance of the disaccharide structure compared with monosaccharide structures for adjuvant activity. In contrast, the fatty acid composition, including stereospecificity, is extremely critical for expression of adjuvant activity of lipid A.[16,17] The number and composition of lipid A fatty acids would be expected to have an important effect on aggregation characteristics of lipid A and on the interactions of lipid A with cells, with liposomes, and with liposomal antigen. Lipid A fatty acids, therefore, might influence the adjuvant effects of liposomal lipid A in vaccine formulations that utilize liposomes as carriers. However, such influences of lipid A fatty acids cannot be easily predicted, and the relative efficacies of lipid A analogs can only be determined by empirical testing in candidate vaccine formulations.

B. ROLE OF MACROPHAGES

Early studies on the fate of radioactively labeled, parenterally injected LPS and lipid A demonstrated that both compounds rapidly left the blood and accumulated in macrophages in the liver and spleen.[18] The concept that the adjuvant effect of LPS was related to its effect on macrophages was at least partly based on experiments involving transfer of murine macrophages

from donor animals to syngeneic recipient animals. Donor macrophages that had been allowed to ingest antigen *in vitro* enhanced the immune response to the antigen in the recipient animals, and enhancement was greatly stimulated by pretreating the macrophages with LPS.[19-21]

The adjuvant effects of LPS and lipid A on macrophages have now been well established. The interactions of LPS with macrophages cause myriad macrophage synthesis and secretory activities, and signal transduction mechanisms that are sometimes referred to as macrophage activation.[22] In addition to the recruitment and activation of macrophages, it is also recognized that LPS and lipid A stimulate multiple cellular interactions. An important role of interactions of macrophages with T-lymphocytes has been proposed as a major basis for adjuvant effects of LPS.[23] The cellular interactions induced by LPS and lipid A are mediated by numerous secreted cytokines. Some of the complex interactions that occur between mediators and cells as a result of LPS and lipid A stimulation are illustrated in Figure 1.

C. EFFECTS OF LPS ON ANTIGEN PRESENTATION

In recent years the concept of the macrophage as an antigen presenting cell (APC) has emerged.[25] A key element in the process of antigen presentation is the participation of major histocompatibility gene complex (MHC) molecules. For induction of an immune response against foreign antigens, class II MHC molecules (also known as Ia molecules) are expressed by the APC, and recognition by helper T-cells of a complex of processed antigen with Ia on the surface of the APC is the initial event leading to an immune response.[25] It has now been demonstrated that LPS can stimulate induction of enhanced Ia expression by murine macrophages.[26-28] Besides recruiting and stimulating macrophages to enhance the immune response against antigens, LPS also elicits macrophages that cause increased antigen catabolism. This latter process can lead to down-regulation of the immune response.[29]

D. EFFECTS ON SUPPRESSOR T-CELLS

The immune response to pneumococcal polysaccharide is greatly amplified by the use of monophosphoryl lipid A (MPL).[30] The enhancing effect of MPL was attributed to the inactivation of suppressor T-cell activity, and this may represent a further adjuvant mechanism for lipid A.

III. VACCINE STRATEGIES

Among the most desirable attributes of a modern adjuvanted vaccine are that the vaccine should be safe and that the adjuvant should increase the specific efficacy of the vaccine to a sufficiently high level that the benefit of the adjuvant outweighs any risk and reactogenicity associated with the adjuvant.[31] Although many of the most successful vaccines have had annoying but tolerable reactogenic characteristics, the evolution of vaccine technology

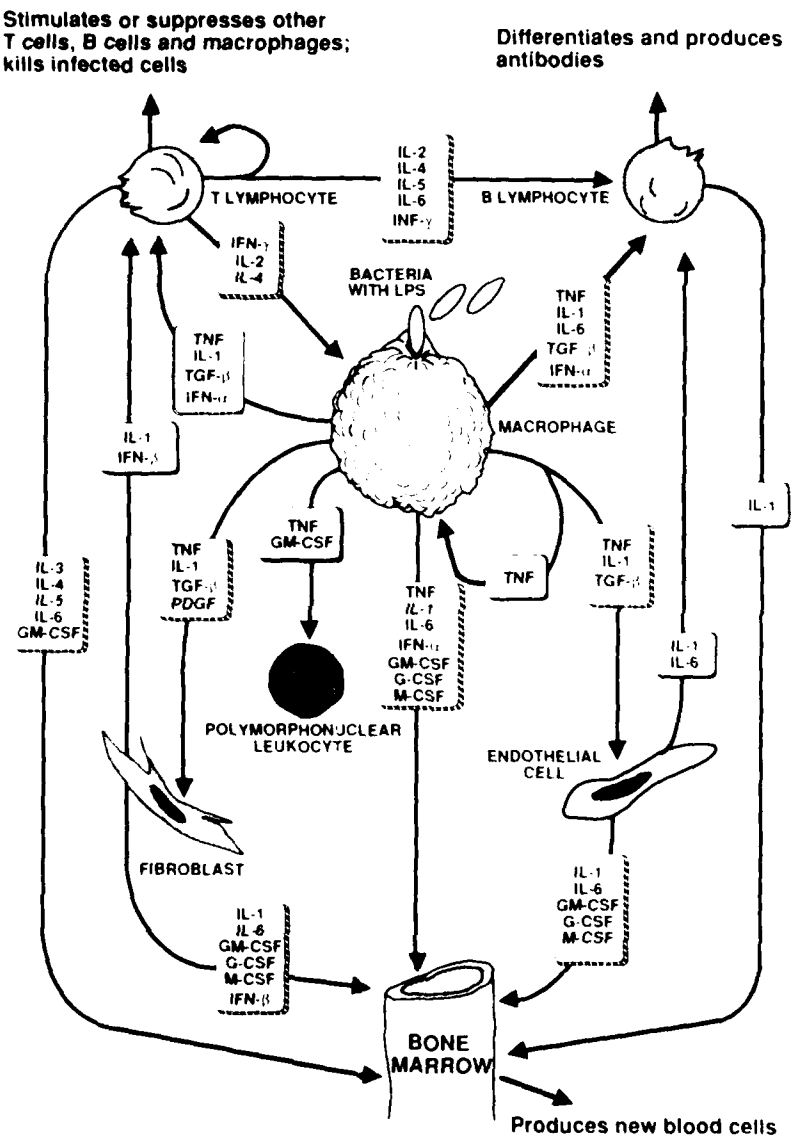

FIGURE 1. Secretion of mediators by an LPS-stimulated macrophage (center) sets in motion further secretory stimuli and biological effects by numerous cell types. Of particular interest for adjuvant development for vaccines are the effects of tumor necrosis factor (TNF), interleukin (IL) 1, IL-2, IL-4, IL-5, and IL-6, growth factor (TGF-β), and interferon γ (IFN-γ), on proliferation and differentiation of T- and B-lymphocytes that are crucial elements in the process of antigen presentation. (Redrawn and based on information from Reference 24.)

has now led to proliferation of myriad peptide and recombinant protein antigens that have inherently high levels of safety because of their purity. Unfortunately, simplified soluble protein antigens do not necessarily have high levels of inherent immunogenicity. In many cases this is because the original antigen is membrane-associated, and in its native form it and its associated neighboring environment are handled by the immune system as a particle. Particulate antigens undergo efficient processing and presentation by macrophages. Soluble antigens are much more likely to be processed by other types of APCs such as B-lymphocytes or dendritic cells. Macrophages, because of their complicated biological roles as scavenger and secretory cells, are more likely to induce potent immune responses to complex foreign antigens.

A. SAFETY

As noted earlier, macrophages are widely believed to serve as the target cell for LPS and lipid A, and also for many other types of adjuvants. Unfortunately, the same properties that stimulate secretory events leading to enhancement of the immune response can sometimes also cause undesirable local or systemic reactions that limit the potential usefulness of LPS and lipid A as adjuvants. One of the most common adverse reactions to vaccines is pyrogenicity, and it is perhaps ironic in the present context to note that in many instances the efficacy of the vaccine may be dependent on the presence of LPS, either as an unintended contaminant or as an integral part of the antigen, and the LPS may also be responsible for induction of pyrogenicity.

It is well known that different biological effects of LPS and lipid A can be related to different chemical moieties in the structure of LPS and lipid A. It has been argued that certain so-called "beneficial" effects may be identified with chemical structures that are different than the structures associated with "toxic" effects.[1] The concept that nontoxic structures might be produced that retain the potent adjuvant effects of lipid A has generated optimism and interest in lipid A among investigators in the field of vaccinology.

1. Lipid A Analogs

Ribi and colleagues have demonstrated that an MPL formulation exhibits reduced lethality and pyrogenicity, but still retains antitumor and adjuvant activities.[2,3,4,5,6,7] In the same context, it has also been a major goal in the lipid A biosynthesis field to produce lipid A analogs that would retain beneficial biological activities, including adjuvanticity, but lack endotoxic activity.[1,2,3,4,5,6,7] Both of these approaches, viz., chemically altered native lipid A and synthetic low toxicity analogs of lipid A, show considerable promise as adjuvants for vaccines. One possible limiting factor to these approaches would be if significant residual reactogenicity of the lipid A formulation were still observed at doses of lipid A that would be required for optimal immune responses. Intravenously administered MPL has been tested in a Phase I trial

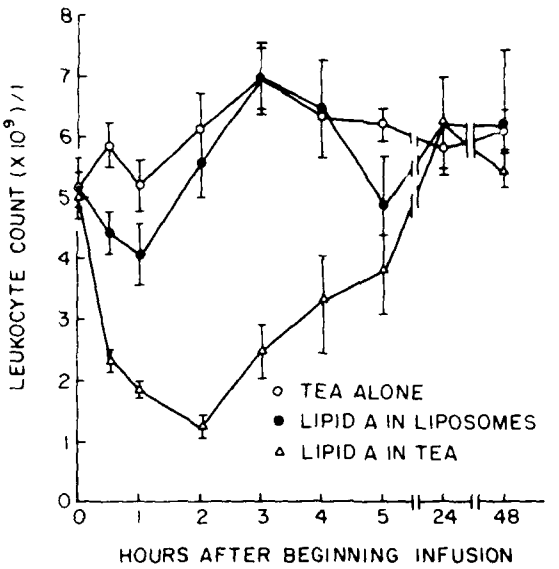

FIGURE 2. Effect of infusion of lipid A (10 μg/kg) on leukocyte counts (mean ± SEM) in rabbits. Lipid A that was in 0.5% triethylamine (TEA), or in liposomes, was diluted with 0.9% NaCl and infused during 1 h into four rabbits. Six controls received 0.5% TEA in 0.9% NaCl. (From Reference 34.)

in humans.[35] The maximal safe intravenous dose in humans was estimated to be 100 μg/m^2, but even at 25 μg/m^2 minor episodes of fever, gastrointestinal symptoms, and chills were often observed.[35] Most vaccine formulations are given intramuscularly or subcutaneously, and it is possible that these latter routes of administration might result in less reactogenicity than the intravenous route.

2. Liposomes Containing Lipid A

Another approach that has been employed in an effort to widen the gap between reactogenicity and adjuvanticity has been to incorporate lipid A into liposomes. Among the activities of lipid A that are reduced by incorporation into liposomes are included: Limulus lysate coagulation *in vitro*,[36,38] neutropenia in rabbits,[36] stimulation of both interleukin 1[38] and tumor necrosis factor[39] by a macrophage cell line, pyrogenicity in rabbits,[40] and lethal toxicity to mice.[41] Intravenously infused lipid A characteristically induces a profound neutropenia in rabbits, with a nadir reached several hours after initiation of infusion. As shown in Figure 2, neutropenia was not induced by liposomal lipid A.

One of the most common complaints associated with many vaccines is the occurrence of fever. In many cases pyrogenic reactions are due to the

TABLE I
Effect on Pyrogenicity of Incorporating Native Lipid A and Monophosphoryl Lipid A in Liposomes[a]

Lipid A type	Maximum nonpyrogenic dose[b]		Decrease in pyrogenicity of liposomal compared with free lipid A
	Free lipid A	Liposomal lipid A	
S. minnesota R595	0.008	1.7	214-fold
MP lipid A (MPL)[c]	0.32	8.1	25-fold

[a] Data obtained form Reference 40.
[b] Maximum nonpyrogenic dose was the maximum lipid A dose (μg/kg) that did not cause an increase in temperature of 0.6°C or greater in any of three rabbits over a 3-h period.
[c] MP lipid A is a monophosphoryl lipid A fraction derived from *Salmonella minnesota* R595 lipid A.

presence of LPS or lipid A, either as a contaminant or as an antigen in the vaccine. As mentioned earlier, MPL is less pyrogenic than native lipid A when tested in a rabbit pyrogenicity model. In experiments designed to explore the roles of physical environment and chemical structure on pyrogenicity, MPL was approximately 40-fold less pyrogenic than the native lipid A from which the MPL was derived (maximum nonpyrogenic dose of 0.32 compared with 0.008 μg/kg) (Table 1). Liposomes further reduced the pyrogenicity by at least 25-fold (Table 1), thus causing liposomal MPL to be 1000-fold less pyrogenic than free native lipid A. Vaccine grade liposomal MPL prepared for human use according to Good Manufacturing Practices (GMP) as promulgated by the U.S. Food and Drug Administration, was 8-fold less pyrogenic yet (maximum nonpyrogenic dose of 66 μg of liposomal MPL per kilogram).[41] Inclusion of aluminum hydroxide (alum) as a nonliposomal adjuvant reduced the pyrogenicity of GMP liposomal MPL still further (approximately tenfold more).[41] It is, therefore, estimated that GMP liposomal MPL adsorbed to alum may be approximately 80,000-fold less pyrogenic than native lipid A alone.

Despite the virtual elimination of pyrogenicity, liposomal MPL still serves as an extremely potent adjuvant for inducing humoral immunity against liposomal protein antigens both in animals[30] (Figure 3) and humans.[4] The previously expressed concern that lipid A in liposomes would serve as an adjuvant only at pyrogenic concentrations, and that lipid A would, thus, be unacceptable for use in humans,[43] has, therefore, been proved to be unfounded. The strong adjuvant effects of nonpyrogenic formulations of liposomal lipid A are also consistent with the observations mentioned earlier that adjuvant effects of certain synthetic lipid A analogs can be dissociated from pyrogenic effects.

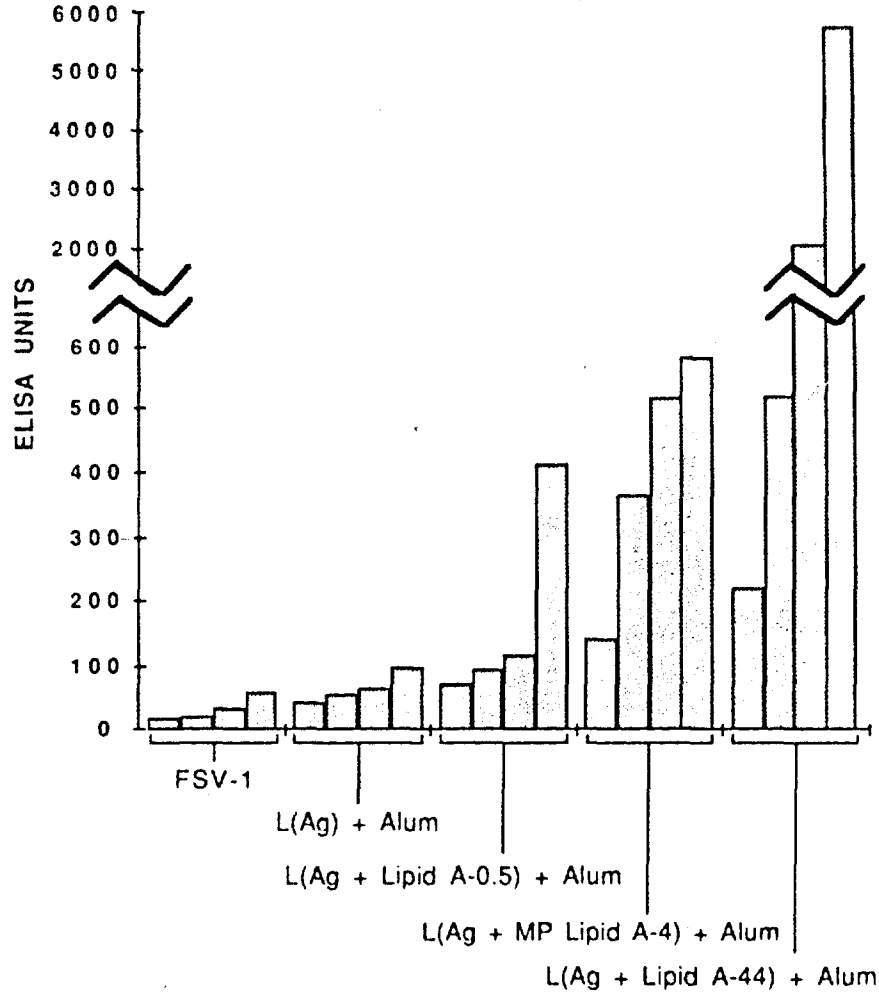

FIGURE 3. Immune response of individual monkeys to liposomes containing a recombinant antigen containing epitopes from the circumsporozoite protein of *Plasmodium falciparum*. The liposomes contained either native lipid A (0.5 or 44 μg/kg, as indicated) or monophosphoryl lipid A (4 μg/kg) as an adjuvant. The liposomes were adsorbed with alum where indicated. The FSV-1 formulation (Falciparum Sporozoite Vaccine-1) consisted of the antigen alone adsorbed to alum, and this was the same vaccine that was previously tested in a human clinical trial. (From Reference 40.)

B. ENHANCEMENT OF IMMUNOGENICITY

In the context of vaccine usage an adjuvant is defined as a substance that enhances the specific immune response against an antigen. Research on the effector mechanisms of various adjuvants has revealed that different adjuvants may work at different stages or locations in the immune response. A rational strategy in formulating a vaccine would, therefore, be to enlist combinations of substances that could exert adjuvant effects at more than one point. This would allow additive effects to occur, and it might permit the usage of smaller amounts of each adjuvant component, thus promoting increased safety.

Because of the central role played by macrophages in adjuvant mechanisms of LPS and lipid A, a logical approach would be to promote increased delivery of LPS or lipid A to macrophages. One of the most effective methods currently being employed to deliver therapeutic substances to macrophages is to use liposomes as carriers.[44-46]

1. Use of Liposomes

Liposomes have been highly successful as carriers of antigens and adjuvants.[47] The theoretical basis for interactions of liposomes with the immune system and for enhancement of immune responses against liposomal protein antigens has been extensively reviewed elsewhere.[47] Liposomes provide a means to reconstitute hydrophobic antigens, or to encapsulate soluble antigens or peptides. They also provide a solid surface for the chemical attachment of antigens.

Intravenously injected liposomes are rapidly removed from the blood and are avidly ingested by macrophages in the liver and spleen where they undergo gradual degradation. The fate of intramuscularly or subcutaneously injected liposomes is dependent to a great extent on the scavenging characteristics of local tissue macrophages or macrophages recruited to the site in response to an inflammatory or adjuvant stimulus. For example, liposomal antigen (adenovirus hexon) that was injected intramuscularly was slowly released from the injection site with a half-life for clearance of 30 to 44 h.[48] It is, therefore, evident that a transient sustained release or depot effect contributes to the mechanism by which intramuscularly injected liposomal antigen interacts with the immune system. The depot effect of liposomes probably does not substantially enhance the adjuvant effect of lipid A compared with that observed with lipid A alone since it has been reported that subcutaneously injected lipid A alone has a half-life at the injection site of more than 15 d.[49]

As noted earlier, liposomes also attenuate the toxic effects of lipid A, but the adjuvant effect of lipid A is retained even in the absence of pyrogenicity or other types of reactogenicity. It is, therefore, possible that the blocking of the toxicity of lipid A by liposomes is partly due to retardation of release of lipid A into the environment and also due to degradation of liposomal lipid A along with the liposomes in macrophages.

2. Adjuvant Effects of Liposomes Containing Lipid A

Early studies demonstrated that lipid A enhanced the murine immune responses against liposomal haptens consisting of lipid derivatives of either 2,4-dinitrophenyl or fluorescein groups.[7,50] It was soon suggested that liposomes containing lipid A (or liposomes containing LPS) could stimulate immune responses against liposomal proteins,[9,51] or even against lipid A as an antigen.[9] Lipid A proved to be such a potent adjuvant that antibodies were even induced against both the liposomes themselves and liposomal phospholipid constituents.[52,53]

Liposomes containing lipid A have been used as adjuvants for enhancing immune responses to cholera toxin;[9,54,56] herpes simplex antigens, including glycoprotein-enriched antigens[57] and a peptide-palmitic acid conjugate;[57] Epstein-Barr virus membrane antigens;[58] *Plasmodium falciparum* sporozoite antigens, including a peptide-protein conjugate,[55] and a recombinant protein;[40,59] an unconjugated 25-amino acid peptide from the active site of acetylcholinesterase;[60] and tumor-associated glycophingolipids.[61]

One of the difficulties that is often encountered in adjuvant research lies in evaluating the relative efficacy of an adjuvant in comparison to other adjuvants. There are no standardized techniques available for this purpose because of the existence of multiple adjuvant mechanisms and immunological techniques. Different chemical and physical structures of adjuvants often preclude standardization of all the different possible variables. Comparative evaluation of adjuvants such as lipid A, analogs of lipid A, LPS, and muramyl dipeptide can be a heroic undertaking.[62] However, some of these difficulties can be overcome with liposomes. Many variables can be simultaneously controlled by utilizing liposomes containing both the antigen and the adjuvant. In one study, direct comparison of the adjuvant effects of liposomal lipid A with liposomal lipophilic MDP derivatives demonstrated that, under the conditions employed, lipid A had stronger adjuvant effects than lipophilic MDP (Figure 4).[54]

3. Liposomal Vaccine

The first injectable liposomal vaccine was administered to human volunteers in a Phase I trial in October 1989.[42] The liposomes contained a recombinant protein antigen having repeat sequence epitopes from the circumsporozoite protein of *P. falciparum*. MPL was included in the liposomes as an adjuvant, and the liposomes containing antigen and MPL were also adsorbed to aluminum hydroxide to prolong the depot effect and provide further adjuvant activity. At the highest dose tested, 2.2 mg of liposomal MPL was administered with each intramuscular injection of vaccine (at 0, 12, and 20 weeks). Despite the extremely high doses of liposomal MPL (approximately 12-fold greater than the maximum safe dose of intravenous free MPL in humans),[35] the liposomal MPL exhibited virtually no acute toxic effects. The results from this phase I human trial have demonstrated that extremely high levels of specific IgG antibodies against the appropriate antigenic epitopes have been achieved.

The success of the human liposomal vaccine in providing exceptionally high levels of serum antibodies validates the concept that lipid A can be combined with other types of carriers and adjuvants to provide additive adjuvant activities. The high doses of MPL employed also demonstrate that potent adjuvant effects can be achieved in the absence of toxicity. Because of the success of this initial vaccine for including enhanced immunity, ad-

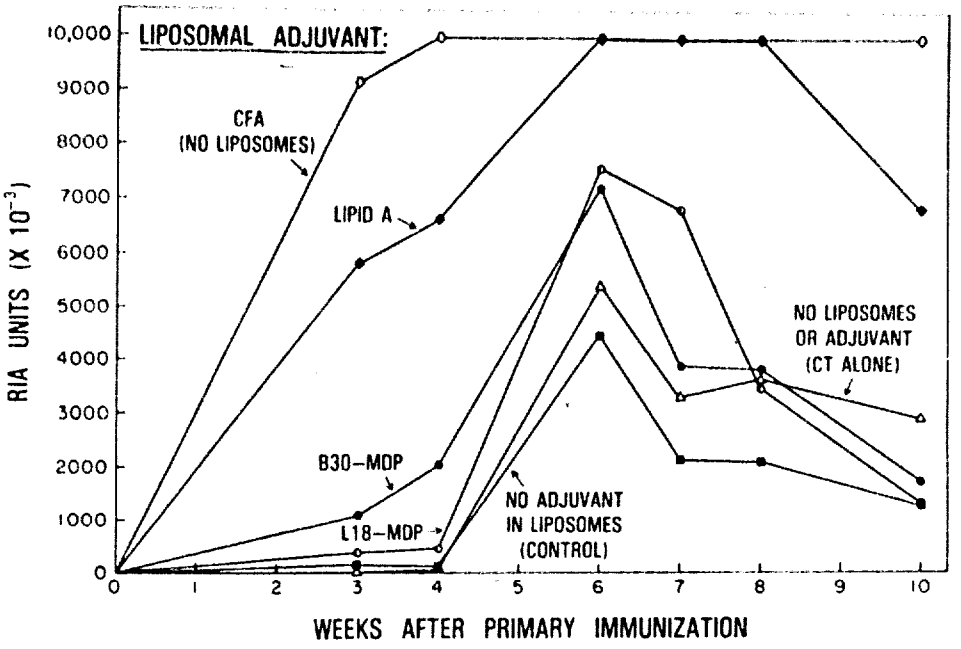

FIGURE 4. Immune response against cholera toxin (CT) alone, CT associated with complete Freund's adjuvant (CFA), liposomes, or liposomes containing lipid adjuvant. Each line is the mean of two rabbits immunized either with 5 μg CT alone (i.v. injection), or with 5 μg CT either emulsified with CFA (s.c. injection), attached to the surface of liposomes with no adjuvant, or attached to the surface of liposomes containing either lipid A or one of two forms of lipophilic MDP, $CH_3(CH_2)_{16}CO$-MDP (stearoyl-MDP, or L18-MDP) or $[CH_3(CH_2)_{13}]_2CHCO$-MDP (B30-MDP) (i.v. injection). The maximum measurable level of radioimmunoassay (RIA) units in the assay was 10^7. Therefore, for those data points for CFA and lipid A in which this latter value is illustrated, the actual values achieved were probably higher. (From Reference 55.).

ditional candidate human liposomal vaccines employing other antigens and MPL have been proposed, and several are now in advanced stages of development for initial clinical trials.

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